Undergraduate thesis: Germination and dormancy of crop-wild sunflower hybrid cross types

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Abstract

Gene flow from agricultural crops into related wild plant populations can produce viable hybrid offspring with characteristics that differ significantly from those of their wild counterparts. Such crop-wild gene flow, especially in situations involving transgenic crops, has raised concerns about potential losses of genetic diversity, or increases in invasiveness, of wild relatives. The annual sunflower (*Helianthus annuus*) is an ideal species for studying crop-wild gene flow, the subsequent hybrid generations produced, and the effects on wild populations because the crop and wild types are often found in close proximity, are cross compatible, share pollinators, and are differentiated for important traits that may affect fitness, such as seed dormancy. Outside of cultivation, seeds that germinate in the fall are killed by the winter frost before reproducing, which has lead to the evolution of dormancy in wild populations; their seeds must experience a cold period before germinating in the spring. By contrast, cultivated varieties have been artificially selected to germinate immediately under favorable conditions. Consequently, the degree to which crop-wild hybrids germinate under fall conditions affects the extent to which crop genes may persist in, or introgress into, wild populations. Furthermore, the germination and dormancy characteristics of sunflower seeds, in particular, are due, in part, to the structural characteristics of their achenes (i.e., seeds and hulls). Most of these structures are maternally inherited while the genetics of the embryo involve contributions from both parents. This project determined the rates of germination and dormancy of fifteen crop-wild hybrid sunflower cross types and used microscopy to explore achene characteristics that could influence the differential germination we observed. Maternal effects (e.g., seed covering structures) had significant impacts on germination and dormancy, with crop-produced achenes germinating most readily, followed by those produced on F1 maternal plants, followed by those produced on wild plants.

The proportion of crop alleles (i.e., nuclear genetics of the embryo) was also important. There was a significant, positive relationship between the proportion of crop alleles and germination in the wild and F1 produced seeds. Microscopy revealed that maternal parent significantly affected achene size, embryo size, and relationships between structures and the micropylar (i.e., pointed) end of the achene. Crop produced achenes were largest and had minimal structural barriers to radicle (i.e, embryonic root) extension. F1 produced achenes were larger and had more substantial barriers which broke down quickly. Wild produced achenes were smallest and had the greatest barriers to radicle extension. Although they differed in size, structural characteristics, and germination rate, seeds produced on crop, F1, and wild maternal parents underwent the same general processes of leading up to germination. These findings suggest that crop alleles and the maternal effects associated with crop-wild hybridization can reduce dormancy, but that this reduction in dormancy may not be significant enough to prevent the introgression of crop alleles into wild populations.

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Introduction

Many crop species can reproduce with related wild species (Ellstrand 2003), yielding hybrid offspring that differ from their wild parents in certain evolutionarily and ecologically important characteristics (Cummings et al. 1999; Snow et al. 2001; Mercer 2006a). In many cases, this gene flow from crop to wild populations may be recurrent, as pollen from crops fertilizes nearby wild populations year after year. Such gene flow may present several significant evolutionary consequences, including extinction by assimilation, reductions of valuable diversity, and increased weediness (Ellstrand 2003). Extinction by assimilation occurs when neutral or beneficial genes infiltrate a population and replace the alleles that previously characterized it as genetically distinct. An example of this effect is the extinction of *Argyranthemum cornopifolium*, a plant species that was endemic to the island of Tenerife until 1996, when it was driven to extinction by hybridization with, and displacement by, its weedy cousin, *A. fructescens*, which had flourished due to human disturbances (Levin 2001, as cited in Ellstrand 2003). Modeling studies indicate that extinction by assimilation may also be a significant threat to wild sunflowers ,which are subject to gene flow from crop sunflowers (Wolf et al. 2001, as cited in Ellstrand 2003).

Even if the assimilation is not complete enough to eradicate the species, there is concern that any reduction of genetic diversity may compromise a species' ability to evolve in response to environmental change and diminish an important resource for crop improvement. Whitton et al. (1997) found that after a crop *Helianthus annuus* was planted adjacent to a natural *H. annuus* population for a single growing season, crop alleles not only persisted in the wild population

throughout the five generation study period, their diffusion outward from the zone closest to the crop planting had the effect of homogenizing the population.

Another concern regarding crop-wild gene flow is its potential to result in the evolution of weedy or invasive species. The introgression of certain advantageous genes may cause a wild relative to evolve into a weed or increase its weediness (Ellstrand 2003; Snow 2001). One example of such weed evolution is the hybridization of *Secale cereal* with *S. montanum*, which became known in California as weedy rye. The new weed was so severe that by 1989, farmers in California had abandoned the cultivation of rye for human consumption (National Academy of Sciences 1989, as cited Ellstrand 2003). Johnsongrass (*Sorghum halepense*) may be another such example. It is one of the world's worst weeds, but it is especially problematic in North America. Weed evolutionists have hypothesized, based on its morphology and development in North America, that the increased weediness here is a result of crop gene introgression from *S. bicolor*.

While these concerns previously existed, the widespread use of transgenic crops has increased their prominence and has prompted questions about whether transgene flow may present uniquely potent ecological dangers, especially with regard to the weediness of wild populations (Burke et al. 2002; Ellstrand 2003; Snow et al. 2003). Transgenes frequently confer increased resistance to herbicides, pesticides, and environmental stresses. Such resistance, if expressed in wild populations, may allow the populations to expand in geographic range and make them more resilient against human control efforts (Ellstrand 2003). Furthermore, transgenes are, in almost all cases, genetically dominant—a condition which facilitates their expression in hybrid progeny

(Whitton et al. 1997; Ellstrand 2003). Researchers have already found evidence for the introgression of transgenes into non-transgenic populations (Watrud et al. 2004; Mercer and Wainwright 2008; Weiger et al. 2011). Strong evidence for the persistence of crop genes in wild populations indicates that the research community must broaden its focus beyond hybridization rates to include new studies on hybrid fitness (Burke et al. 2002).

Research on the fitness of crop-wild hybrids is crucial because, although the impacts of crop-wild gene flow are potentially serious, they are also highly dependent on the environmental and biological context. The fate of crop genes once they move into wild populations depends on whether they are integrated into the wild population (i.e., introgressed) or quickly lost. This will depend on their effects on fitness, i.e., whether they have positive, neutral, or negative effects (Ellstrand 2003). Thus, introgression is contingent upon local ecological conditions and the genetic background of specific wild populations (Mercer et al. 2006b). For example, a transgene for herbicide resistance would be more advantageous in a population that is regularly exposed to herbicide than in one that is not. Patterns of dormancy and germination may also influence crop gene introgression. Increased germination rates in hybrids could hasten crop gene introgression as long as the hybrids germinate at the appropriate time (Mercer et al. 2006a). Conversely, if the environment is seasonally cold, reduced dormancy in crop-wild hybrids could result in fall germination—an evolutionary dead end since and plants would be killed by the cold of winter before reproducing.

The common sunflower (*H. annuus*) is an ideal species for studying crop-wild gene flow, as well as the characteristics of subsequent hybrid generations, because crop and wild types are often

found in close proximity, are cross compatible, and share pollinators (Burke et al. 2002). Thus, there are numerous crop-wild hybrid zones across on the landscape. Sunflower has also been well studied. It is known that crop genes exist in wild populations (Whitton et al. 1997; Burke et al. 2002), and a significant amount is known about the ways crop genes affect fitness-linked characteristics of wild populations (Snow et al. 1998; Cummings et al. 2002; Mercer et al. 2006a, 2006b, 2007). An experimentally introgressed transgene has even been shown to increase seed production and decrease herbivory in wild sunflower (Snow et al. 2003). In addition, crop and wild sunflowers are differentiated for important traits, such as seed size and dormancy (Mercer et al. 2006a, Wills and Burke 2007). *Helianthus annuus* is ideal for studying the implications of gene flow on fall germination in particular because it is indigenous to North America (Blackman et al. 2011), where a temperate climate with large seasonal fluctuations enforces severe fitness consequences for seeds that germinate at an inappropriate time. Although some foundational research has already been conducted to suggest general causes of dormancy in sunflower (Finch-Savage and Leubner-Metzger 2006; Brunick 2007), these causes remain to be studied in greater depth (Walters 2006), especially with respect to how they may differ along the continuum of hybrid cross types that lie between the non-dormant crop and the dormant wild relative. This project contributes to the understanding of crop gene introgression and its possible effects by studying the germination and dormancy patterns of an unprecedented number of sunflower cross types that may exist in hybrid zones and identifying physical mechanisms that may be responsible for these differences.

Because the introgression of crop alleles into wild sunflower populations is partially dependent on the appropriate germination of crop-wild hybrid seeds, elucidating the process of germination

and the causes of dormancy in *H. annuus* is crucial to understanding the implications of crop-wild gene flow within the species. Germination is the process by which a dry, dormant seed increases in metabolic activity, initiates the transformation of the embryo into a seedling, and culminates in the protrusion of the radicle (i.e. embryonic root) (Aliotta and Cafiero 2001). Dormancy, then, is a biological phenomenon that blocks a viable seed from germinating under otherwise favorable conditions. Finch-Savage and Leubner-Metzger (2006) classify *H. annuus* as having non-deep physiological dormancy, which means that excised embryos produced normal seedlings. Non-deep physiological dormancy can be broken by treatment with giberellins, and sometimes by scarification, after-ripening in dry storage, or stratification in cold or warm storage. Physiological dormancy is also discussed in terms of the structures within the seed that are responsible for the seed dormancy, such as embryo dormancy, testa dormancy, and pericarp dormancy (Finch-Savage and Leubner-Metzger 2006; Brunick 2007).

The structural anatomy of sunflower seeds has been well studied (Roth 1970; Schneiter 1997), although research on how each of these anatomical structures affects dormancy in different crop and wild varieties of *H. annuus* is just beginning (Brunick 2007). Sunflower achenes can vary greatly in shape and size. Wild achenes can be as short as 2 mm and as narrow as 1mm, while crop achenes can be as long as 25 mm and as wide as 13 mm (Schneiter 1997). Crop-wild F_1 hybrids tend to produce achenes with an outward appearance that is intermediate between crop and wild achenes (Weiss, personal observation). The nutlike fruit of the sunflower, commonly referred to as a seed, is known to botanists as an achene. Two basic parts of the achene are readily visible without magnification: the hard, dark hull, formally referred to as the pericarp, and the lightly colored kernel, which is the true seed (Schneiter 1997).

Magnification reveals more intricate structures. The pericarp is actually covered in small, twin hairs, up to about 560 µm long. These hairs grow out of the outermost layer of the pericarp, known as the epicarp, which is made up of longitudinally elongated cells. The epicarp cells can be up to about 200 µm across. Their walls may be pitted and may contain pigments, giving the achenes a dark or striped appearance. Also within the pericarp, but below the epidermis, lies the hypodermis, which consists of regular cells with pitted walls. Just within the hypodermis is the non-cellular, dark brown or black phytomelanin layer. The innermost layer of the pericarp consists of parenchyma tissue with large intercellular spaces (Vaughan 1970). Below the pericarp is the seed coat, also known as the testa. The testa is very thin and consists of three layers. The inner and outer layers are parenchymal, but the layer in between is spongy parenchyma (Schneiter 1997). The endosperm is coalesced to the inside of the testa and is made up of aleurone cells (Schneiter 1997). These cells are 15 to 65 µm in diameter, and the endosperm layer is only one or two cells thick (Vaughan 1970). The innermost part of the achene is the emryo. Both the blunt and pointed ends of the embryo are adjacent to air cavities (Schneiter 1997). At the pointed, or micropylar, end is the radicle, which is the embryonic root (Schneiter 1997). It must protrude through the embryo covering layers for the seed to germinate (Aliotta and Cafiero 2001). The blunt, or chalazal, end of the embryo consists of two cotyledons which spread apart bilaterally after the seed germinates (Scheiter 1997).

All of these achene structures may influence dormancy and germination in sunflower (Brunick 2007). Although only preliminary research has been conducted on the effects of various achene structures on the germination of sunflower in particular, seed coverings (i.e., testas and

pericarps) are known generally to block germination through a variety of mechanisms. They can mechanically restrain the radicle (Finch-Savage and Leubner-Metzger 2006), prevent water from reaching the seed (Hu et al. 2008; Rathjen et al. 2009), filter the light that reaches the embryo, inhibit respiratory gas exchange, contain chemical inhibitors, block the escape of inhibitors from embryo, or any combination of these (Gosling 2006). The embryo itself can also have dormancy, failing to grow even when its coverings are removed (Finch-Savage and Leubner-Metzger 2006; Brunick 2007).

Due to the nature of achene development, some achene structures represent contributions from both the maternal and paternal parents, while others are strictly maternal. Pericarp development initiates regardless of whether the achene is fertilized, and it is therefore a completely maternal contribution. The testa is also a maternal contribution, as it develops directly from the ovule teguments. The embryo is a product of both maternal and paternal contributions because seed formation begins with the union of male and female nuclei to form a zygote. Although the nuclear genetics of the embryo reflect approximately equal contributions from both parents, its size, at least in sunflower, appears to be dictated by the size of the maternally-inherited pericarp. Regardless of paternal parent, embryos tend to grow until they reach the lateral walls of their pericarps and do not outgrow their pericarps during seed formation (Weiss, personal observation). After embryo formation begins, three additional nuclei, one paternal and two maternal, begin endosperm formation (Scheiter 1997; Aliotta and Cafiero 2001). In sunflower, endosperm development is very limited (Vaughan 1970; Finch-Savage and Leubner-Metzger 2006). As the achene development process suggests, the genetics of both parents are involved in achene formation, but the maternal contribution is more substantial.

Due to the disparity between the maternal and paternal influences on seed formation, it can be useful to distinguish the contributions of two heritable factors on dormancy: embryo nuclear genetics and maternal effects. The nuclear genetics of the embryo are a product of equal contributions from both parents. In reference to crop-wild hybrids, nuclear genetics of a given cross type or hybrid generation can be summarized by the average percentage of alleles of crop origin versus those of wild origin that an embryo may possess. For example, sunflower seed produced on a crop head, pollinated by a wild plant, will possess alleles that are 50% of crop origin and 50% of wild origin. That F1 hybrid, if pollinated by a wild plant, will produce F1×W seed with approximately 25% alleles of crop origin and 75% alleles of wild origin. Maternal effects are those characteristics of a seed that depend only on the identity of the maternal parent. Maternal effects encompass a range of factors, including maternal inheritance of the cytoplasm (and organellar genetics), the stronger dose of maternal genes in the endosperm, and, possibly most importantly with respect to germination, the testa and pericarp the maternal plant imparts. For example, the embryo covering layers of an achene produced on a crop head will be the same regardless of whether the head is pollinated by a crop plant or a wild plant (Weiss, personal observation). Previous research investigating effects of crop-wild gene flow on seed dormancy, using six hybrid cross types which were germinated with and without stratification suggests that such maternal effects may be the primary factor affecting variation in germination and dormancy, while the percent of alleles of crop origin (hereafter percent crop alleles) is an important secondary influence (Primer and Mercer, unpublished data). This project explores this idea further by testing a greater number of cross types that may occur in hybrid zones, focusing specifically on fall germination (i.e., germination without stratification), and observing the

physical processes that may be responsible for differences in dormancy and germination among different cross types.

This project had three objectives:

1. To determine the degree to which achenes germinate, remain dormant, and die under simulated fall conditions for fifteen cross types of *H. annuus* that vary in nuclear genetics (i.e., percent crop alleles) and maternal parent
2. To discern the degree to which nuclear genetics and maternal effects are responsible for differences in fall germination among our cross types.
3. To investigate how the physical structures of achenes, especially those that are maternally inherited, change during the imbibition and germination processes.

I achieved these objectives by first quantify the germination and dormancy of a wide variety of sunflower achenes with known pedigree and then studying the dormancy controlling achene structures of different sunflower cross types as the achenes imbibed and germinated. This experiment quantified the germination and dormancy of an unprecedented number of cross types, as compared to related studies of sunflower germination (fifteen vs. six or three). The seeds spanned the range from 0 to 100% crop alleles (variable nuclear genetics) and were produced on wild, F_1 hybrid, or crop plants (variable seed coverings and other maternal effects). By testing fifteen different cross types, with varying maternal effects and percent crop alleles, I was able to discern the degree to which each of these two factors influence fall germination rates and, thus, fitness. In addition to observing the cumulative effects of both factors, I drew direct comparisons between discrete cross types with the same percent crop alleles but different maternal types (e.g., crop×F_1 vs. F_1×crop) and cross types with the same maternal types but different percents of crop alleles (e.g., F_1× wild vs. F_1× crop). I also used dissection,

microscopy, and digital image analysis to explore the maternal effects that differentiate three of the cross types tested that have 50% crop alleles but different maternal parents (i.e., crop×wild, F_1× F_1, and wild×crop). Analyses of the results of this imaging study in conjunction with the results of the germination quantification study offer a clearer picture of the physical mechanisms that underlie differentiated fall germination and thus influence crop gene introgression.

Hypothesis

I expect to observe a relationship between cross type and probability of germination. I anticipate that effects of the maternal parent will dominate, with percent crop alleles having a lesser, but still significant, influence. I expect seeds produced on crop plants to have the highest germination, followed by F_1-produced seeds, followed by wild-produced seeds. I also expect to see a positive correlation between percent crop alleles and germination, with cross types that have higher percent of crop alleles, within a given maternal type, germinating more readily. Relationships between characteristics of embryo covering structures and germination rates are also expected. Differences between maternal types in structures at the micropylar end of the achene are expected to be particularly significant because this is the end from which the radicle emerges, completing the germination process. I anticipate that wild pericarps will be a more significant barrier between the embryo and the environment surrounding the achene, sealing out more water and limiting radicle extension for a longer time period than crop pericarps. F1 seed coverings will exhibit characteristics that are intermediate to the crop and wild types.

Materials and Methods

Germplasm and Crosses:

The achenes studied were produced through hand-pollinations at the Ohio State University's Waterman Farm during the 2010 growing season. Achenes from USDA inbred line HA89 were used as the crop parent (C), and wild parents (W) came from collections made around Lawrence, Kansas, an area without crop sunflower cultivation but that is in proximity to possible hybrid zones. Wild, Crop, F1, F2, and backcross (BC) parents had been produced in 2009 from the same materials. Fifteen cross types were produced in total (Table 1), produced on three maternal cross types and resulting in eight levels of percent crop alleles. Of those fifteen, three cross types were selected for dissection microscopy. These three cross types represented three maternal types, all with 50% crop nuclear, embryonic genetics (i.e. WxC, F1xF1, and CxW) (Fig. S1).

Prior to each experiment achenes were bulked by cross type. I used achenes from a variety of maternal and paternal parents to render the experiment more representative of the mating that may occur in actual hybrid zones. Eight maternal families and two pollen parents per maternal family were bulked for each cross type when possible, except for the CxC cross type, which were produced through eight self-pollinations. Limitations on the types and number of achenes available restricted the number of maternal and paternal parents used for some cross types (Table S1).

Experiment 1: Germination rates under simulated fall conditions

The objectives of this experiment were to determine the degree to which the fifteen cross types germinated, remained dormant, and died under simulated fall conditions, and to discern the degree to which nuclear genetics and maternal effects are responsible germination differences between the cross types. This experiment simulated fall germination without prior cold stratification (i.e., without a treatment to break dormancy) using a climate-controlled growth chamber (Conviron G30). Growth chamber settings were chosen to simulate average October climate data from Lawrence, KS, where the wild germplasm originated (Table 2).

After bulking by cross type, achenes were washed in a 10% bleach solution and rinsed well to limit fungal growth during the germination period. Then, 20 seeds were then placed in each petri dish lined with moistened blotter paper. Crop x wild petri dishes contained fifteen seeds due to limitations on the number of available seeds. Petri dishes were arranged in a randomized complete block design with growth chamber shelves as blocks. As such, each of the ten replicate shelves contained fifteen petri dishes, one for each cross type. The growth chamber experiment ran for 27 complete days. Every day, the growth chamber temperature was verified, and the blotter paper was moistened as necessary. Every other day, all germinated seeds were recorded and removed. At the end of this period, the remaining seeds were removed from growth chamber and subjected to a standard tetrazolium viability test to determine whether they were dead or had remained dormant (Delouche et al. 1962). Data were analyzed in SAS GLIMMIX and graphed using SigmaPlot and Microsoft Excel. Overall ANOVA analyses assessed the effects of maternal type and percent crop alleles on percent of viable seeds germinated (i.e. seeds germinated/(total-achenes without embryos-achenes lost)), as well as their interactions after 9,

19, and 27 days. Within each maternal type, the effect of percent crop alleles was also determined using a linear regression model, for the same there time periods. Also, for days 9 and 19, the effects of maternal and paternal parents, as well as their interaction were studied using an additional SAS GLIMMIX ANOVA analysis. Finally, four additional sub-analyses evaluated the effects of maternal type, percent crop alleles, and maternal type × percent crop alleles interactions, on days 9 and 19, for groups of four cross types for which two shared the same percent crop alleles, but had different maternal parents, and two shared the maternal parent, but had different percent crop alleles.

Experiment 2: Dissection Microscopy of Imbibition and Germination

The objective of the second experiment was to investigate how the physical structures of achenes, including those that are maternally inherited, change during the imbibition and germination processes. Because identifying trends is only the beginning of understanding ecological phenomena and a thorough understanding of the underlying mechanisms is also essential, the second experiment focused on identifying morphological characteristics of sunflower seeds that may have influenced the germination patterns observed in experiment one. Dissection, light microscopy, and digital image analysis were employed to study the shapes and sizes of different achene components, as well as how structures changed over time as the achenes were exposed to water. Special attention was paid to characteristics of the seed coverings because they are inherited maternally, and preliminary evidence suggests that maternal effects may have a particularly large influence on sunflower seed dormancy (Primer and Mercer, unpublished data). Therefore, experiment two focused on three cross types of crop-wild hybrid seeds that had the same approximate percent of crop alleles in the embryonic nuclei, but different

types of maternally inherited seed coverings. The crop x wild, F_1 x F_1, and wild x crop cross types were studied because they all have 50% crop genes, but their seed coverings are crop, F_1, and wild respectively (Table 1; Fig. S1).

Achenes were bulked by cross type, as in experiment one. Prior to the experiment, all equipment, including the inner surfaces of the growth chamber, was cleaned with a 10% bleach solution. The achenes themselves were not sterilized.

Using a split plot design, in each of three blocks (germination boxes), 11 achenes of each cross type were organized in rows (i.e., one cross type per row). The positions of these main plots or rows (i.e. back, middle, or front) were randomized for each block. Within each row, 10 achenes were randomly assigned to one of 10 observation times, and one achene was designated as an extra to use in case one of the other achenes was missing its embryo. Thus, an individual achene comprised a subplot. Achenes designated to the 0 hr observation time were set aside to be observed dry. Each germination box was filled with1 cm of water-saturated sand, and was covered with a tight fitting lid and a square of clear plastic wrap, secured by a rubber band. The boxes were then placed in a growth chamber set to constant, favorable germination conditions (i.e. 20°C and dark). Sand was remoistened as needed during the course of the experiment. Achenes were observed 10 times after 0, 6, 12, 36, 60, 84, 108, 132, 156, and 180 hrs in the growth chamber. In total, 385 images were taken and used to assess nine types of measurements on each achene. All observations were conducted using a stereo microscope (Omano SZMN Series), a microscope specific digital camera (OptixCam Summit Series 3.0), and image analysis software (OptixCam OCView), calibrated appropriately for the magnification levels used (all Microscope.com LLC, Roanoke, VA). All measurements were recorded in mm.

Each achene was first imaged whole, under low magnification, laying flat on one of its two slightly rounded faces. Achene width (AW) across the widest part of the achene (Fig. S2) was measured on this image. For germinated achenes, radicle width (RW) perpendicular to the sides of the radicle (Fig. S3) was also measured. In cases where the achene was very recently germinated and only the pointed tip of the radicle had emerged RW values were excluded from analyses. If the cotyledons were still confined within the pericarp, RW was measured directly below the tip of the pericarp. If the cotyledons had expanded out of the pericarp, RW was measured below the cotyledons. Seedlings that had completely shed their pericarps were excluded from measurement.

The achene was then bisected from pointed (micropylar) to blunt (chalazal) end through the middle of its two slightly rounded faces, perpendicular to the ridge-like seam that splits open during germination. The cut surfaces of each of the two halves were imaged separately under low magnification. These images were used to measure achene length (AL) and depth (AD) (Fig. S4), and embryo length (EL) and depth (ED) (Fig. S5). Achene length was measured from the center of the chalazal end to the lightly colored scar at the micropylay end, and excluded any asymmetrical pericarp tissue protruding on either end of the scar. AD and ED were measured at their widest points, and EL was not measured on germinated achenes.

Additional images were taken of the pointed ends of both halves under high magnification, in order to take two more measurements of non-germinated achenes. The first of these measurements was the distance between the tip of the radicle and the outside of the pericarp

(DRP) (Fig. S6). This is equivalent to the theoretical distance that the radicle would need to extend to be considered germinated. When the seam connecting both faces of the pericarp was still at least partially intact, its length was also measured (Fig. S7). This measurement was called pericarp seam length (PSL). For achenes whose pericarp seams had completely deteriorated (i.e. PSL = 0 mm.), the distance between the two halves of the pericarp, called the pericarp gap width (PGW) was measured (Fig. S8). These measurements were standardized by dividing by the most relevant metric of achene size (i.e. DRP/AL, PSL/AL, and PGW/AD) (Table S2).

Final values for each achene were calculated by averaging measurements for each half. Data analyses were based on these averaged, per-achene values. Linear regressions were performed in SAS GLIMMIX to determine the effect of maternal type on these achene characteristics, as well as their relationships with time, and any interactions between maternal type and time. Due to the split plot design, maternal type was tested with the interaction between maternal type and block as the error. Additional regression analyses were performed to determine the direction and strength of the relationship between time and the response measures for each cross type separately. Images were also analyzed qualitatively by arranging images for each cross type in order of developmental stage. These series of images, in conjunction with the quantitative analyses, were used to elucidate the general physical processes that lead to germination.

Results

Experiment 1: Germination Rates under Simulated Fall Conditions

Maternal type, percent crop alleles, and the maternal x crop alleles interaction were all highly significant ($P \leq 0.0003$) in determining the proportion of viable seeds germinated on days 9, 19, and 27 (Table 3). The three different maternal types varied in their patterns of germination over

time. Throughout the course of the experiment, germination was highest for achenes from crop maternal parents; germination of achenes produced on F1 plants was intermediate; and germination of wild-produced achenes was lowest. These differences between maternal types were greatest early in the study and eventually decreased as all maternal types approached high levels of germination (i.e. greater than 80%) (Fig. 1). However, due to variability within maternal types, some maternal types did not differ significantly from each other during the latter half of the experiment according to a Tukey-Kramer adjustment for multiple comparisons (Table 4.)

Similar ANOVA analyses conducted on data from days 9 and 19 that tested the effect of paternal parent instead of percent crop alleles indicated that the effects of the maternal parent, the paternal parent, and the interaction between maternal and paternal parent were also all highly significant ($P < 0.0001$) in determining the proportion of viable seeds germinated (Table 5; Fig 2).

At all three time periods tested (i.e., day 9, 19, and 27) linear regression analyses by maternal types indicated that the effect of percent crop alleles was significant for the wild and F1 maternal types ($P < 0.05$) but not for the crop maternal type (Table 6). The relationship between germination and percent crop alleles was consistently positive for the wild and F1 maternal types but insignificantly negative for the crop maternal type, which exhibited very high germination for all five cross types (Fig. 3).

Four subanalyses evaluated the effects of maternal parent, percent crop alleles, and the maternal × crop alleles interaction on percent viable seeds germinated on days 9 and 19. Although the analysis on day 9 with wild and F1 maternal achenes that had 25% or 50% crop alleles indicated

that the effect of the maternal parent (i.e. seed coverings) was significant, the same comparison performed on day 19 data revealed no significance (Table 7; Fig. 4). A similar comparison on day 9 of F_1- and crop-produced achenes with 50% or 75% crop alleles indicated that effects of percent crop alleles, maternal parent, and the interaction between maternal parent and percent crop alleles were all significant, while the same comparison performed on day 19 showed that only the interaction between maternal parent and percent crop genes was significant (Table 7; Fig 4).

At the end of the germination period (i.e., day 27), the percentages of seeds germinated, dead, and remaining dormant were different for the different cross types (Fig. 5). Out of the seeds that did not germinate, more of the wild-maternal seeds were still dormant, and more of the crop-maternal seeds were dead. F1-produced seeds exhibited an intermediate behavior. Some cross types had more dormancy like the wild-produced seeds; some had less dormancy like the crop-produced seeds; and the cross types with greater percent crop alleles tended to have a lower percent of seeds still dormant.

Experiment 2: Dissection Microscopy of Imbibition and Germination

A qualitative analysis of the images taken revealed that several physical processes occur within sunflower achenes of all maternal types, before they germinate. These processes were not strictly sequential. Some appeared to overlap, occur simultaneously, or occur in a different sequence from achene to achene. One of the first events was the uptake of water, or imbibition (Fig. 6). This change was visible as the cut face of the embryos changed from dry and matte to wet and glossy. Achenes also began the germination process with some amount of loose tissue at the micropylar end. This tissue appeared to disintegrate, as well, clearing the path through

which the radicle ultimately emerged (Fig. 7). Another requisite process was the splitting apart of the "pericarp seam" which connects the hard outer layers of the pericarp on either half of the achene, so the radicle could pass through (Fig. 8). Finally, as the embryo enlarged, the radicle extended outward, ultimately pushing through the "pericarp gap" and growing beyond the bounds of the pericarp (Fig. 9).

ANOVA analysis of the quantitative data, using SAS GLIMMIX, indicated that the effect of maternal parent was significant ($P < 0.05$) on all measurements, except DRP, which for which the maternal effect only became significant after standardization. The effect of hour was significant on AD, EL, ED, DRP, DRP/AL, PGW, and PGW/AD. The maternal × hour interaction was significant on EL, ED, DRP, DRP/AL, PGW, PGW/AD, and PSL (Table 8).

For all seed metrics studied, achenes of the F1 maternal type (F1xF1) had mean values in between those of crop and wild maternal types (CxW and WxC, repectively). F1-produced achenes and embryos were approximately one and a half times as large (in terms of AW, AD, AL, EL, and ED) as wild-produced achenes, and crop achenes were roughly twice as large as wild-produced achenes. For these measurements of achene size and embryo size, all three maternal types were significantly different from each other, according to Tukey-Kramer adjustments for multiple comparisons. Crop-produced achenes had significantly larger (about 1.5× larger) RW than F1-produced achenes. RW of wild-produced achenes was excluded from this comparison of means, because only one wild-produced seed germinated during experiment two (i.e., only one data point available). Although the F1 maternal type was also intermediate between wild and crop maternal types for micriopylar end measurements (i.e., DRP/AL, PGW, PGW/AD, PSL, PSL/AL), some differences were not significant after Tukey-Kramer

adjustments due to variation within maternal types. On average, crop-produced achenes had the longest DRP. But this pattern was reversed when DRP was standardized for AL, when it became largest for the wild-produced achenes. PGW was greatest for crop-produced achenes, even when the measurement was standardized by achene depth AD. Wild-produced achenes had the longest PSL, followed by F1, followed by crop. The same order was present when PSL was standardized for AL (Table 9).

Linear regression analyses by maternal type with SAS GLIMMIX demonstrated that, within the F1 maternal type, the effect of hour was significant for DRP, DRP/AL, PGW, and PGW/AD. Standardized and non-standardized DRP decreased over time, while standardized and non-standardized PGW increased. Within the wild maternal type, the effect of hour was significant on ED, which increased over time. The effect of hour on AD was significant within both the F1 and wild maternal types, and it increased over time (Table 10; Fig. 10).

Discussion

Maternal parent largely determined levels of seed germination in sunflower crop-wild hybrids, with seeds produced on crop maternal parents germinating most readily; wild-produced seeds germinated least; and F1-produced seeds exhibited intermediate germination. Germination also increased as percent crop alleles in the embryo increased within the wild and F1 maternal types. On day 9, a 50% increase in percent crop alleles was linked to a 21% increase in germination for wild-produced seeds and a 29% increase in germination for F1-produced seeds. On day 19, increasing percent crop alleles by 50% increased germination by 21% and 20% for wild- and F1-produced seeds respectively, the same increase in percent crop alleles was linked to a 7% increase for wild-produced seeds, and a 16% increase for F1-produced seeds (Fig. 3). Within the

crop maternal type, there was not a significant effect of percent crop alleles. The differences in germination between maternal types appear may be related to the maternally inherited structures that cover sunflower seeds, such as the pericarp. Crop-produced achenes were the largest, followed by F1-produced, followed by wild-produced. Embryo size varied similarly, which may facilitate faster pericarp splitting and radicle emergence in larger achenes. By contrast, the pericarps of wild-produced achenes presented more substantial, longer lasting barriers to germination. For example, the standardized PSL of wild-produced achenes was approximately 3 times greater than that of F1-produced achenes and 6 times greater than that of crop-produced achenes (Table 9), and it decreased little over time (Fig. 10). These findings suggest that seeds containing a greater percentage of crop alleles and those with crop or F1 maternal parents are more likely to germinate quickly under favorable conditions, a tendency that could decrease their fitness by causing them to germinate during an inappropriate time of year. Nevertheless this characteristic is far from a complete barrier to crop gene introgression. Even some achenes with 75% crop alleles, and crop-inherited seed coverings remained dormant after 27 days of exposure to simulated fall conditions. This study implies that land mangers and biotechnology risk assessors must not depend on the reduced dormancy imparted by crop alleles and crop or F1 maternal effects to preclude crop gene introgression into wild populations.

Maternal effects vs. embryonic nuclear genetics

As expected, maternal type was an important determinant of percent germination. Seeds produced on crop maternal plants exhibited the greatest germination, followed by F_1, followed by wild, and the maternal effect remained highly significant throughout the 27 day study period, even though seeds of all cross types approached high levels of germination by the end of the

study (possibly due to after-ripening or stratification during the course of the experiment; Essay S1). Such significance throughout the course of a 27 day germination period suggests that maternal effects may be the primary factor in determining the germination patterns of sunflower seeds across the crop-wild spectrum.

Embryo genetics was an important secondary factor in determining germination, indicating that the paternal parent also played an important role. For the wild and F1 maternal types, germination increased significantly as percent crop alleles increased, supporting the hypothesis that crop alleles in the nuclei of sunflower embryos can hasten germination (Mercer et al. 2006a). Within the crop maternal type, however, germination decreased slightly as percent crop alleles increased. Although this negative relationship was not significant, it may suggest hybrid vigor in the crop-produced seeds carrying wild alleles. All cross types within the crop maternal type had already reached high levels of germination by day 9, the first time point at which the relationship was assessed. Such high germination may have dampened this trend.

With regard to seeds that did not germinate during the study period, wild-produced seeds were more likely to have not germinated because they were dormant, while most of the crop-produced seeds that did not germinate were dead. F_1 produced seeds exhibited an intermediate pattern of dormancy and death, with cross types containing more wild genes generally exhibiting more dormancy, and cross types with more crop genes having more dead seeds. This observation supports the hypotheses that seeds with more crop alleles and achenes crop- and F1-inherited seed coverings would have less dormancy.

Achene structures and germination

This study also elucidated several important physical characteristics of sunflower achenes that affect germination processes. High magnification inspection of the micropylar end of the achene clearly illustrated that the hard, outer layer of the pericarp is not one continuous structure, but rather two shield-shaped plates adhered to each other along a ridge-like seam. These plates must split apart in order for germination to be complete. In addition to the confluence of these two hard faces of the pericarp (i.e., pericarp seam), the micropylar end of the achene also houses a significant amount of loose obstructive tissue between the hard outer pericarp and the radicle. This tissue too must yield to the extending radicle. Although these general characteristics of achenes are present for crop, F1, and wild pericarps, they are present to different extents, which may explain some maternal affects I observed in my germination study.

Almost all of the crop-produced achenes observed under the microscope, even those that were still dry, already had a split between the two hard faces of the pericarp (i.e. PSL=0 and PGW > 0). This gave them a start on germination because they were already more open to air and water infiltrationfrom outside the achene. On some crop-produced achenes, the tissue next to the pericarp gap appeared red or brown, suggesting that those tissues may have long been exposed to outside air, which oxidized them like apple slices browning when they sit out too long (Fig S9). In contrast, F1- and wild-produced achenes started with their pericarps fused, adding an extra step in the germination process, thus slowing it down. Wild pericarps remained fused longer than their F1 counterparts, which is consistent with the observation that seeds in wild pericarps take longer to germinate. Further study should be conducted on what holds the pericarp seam together and what causes it to split, allowing the pericarp gap width to widen. I hypothesize that

the pericarp seam is initially bound together with adhesive tissue that dissolves when exposed to water and enzymes produced by the seed. Simultaneously, the seed imbibes, and the cotyledons expand outward, splitting the pericarp seam and forcing the pericarp gap to widen. If this force is insufficient, the radicle may also exert force at the miropylar end of the pericarp.

The obvious effect of maternal type on achene size may also have direct implications for imbibition and germination. F1 achenes were roughly one and a half times as large as wild achenes, and crop achenes were about twice as large as wild achenes, for any given linear metric (i.e, AL, AW, AD). Volumetrically, F1 achenes were about four times larger than wild achenes crop achenes were about 14 times larger than wild achenes (volumes estimated using the formula V=AL×AW×AD). All achenes were within the size range described by Schneiter (1997). (I.e., wild achenes were greater than 2 mm in length and1 mm in width, and crop achenes were less than 25×13 mm.) Hernandez and Orioli (1985) compared small and large sunflower achenes produced from the same hybrid cultivar (i.e. nearly genetically identical) and found that large achenes imbibed more quickly in the first 15 hrs after exposure to water, but that small achenes germinated more quickly during the first 38 hrs, of exposure to favorable germination conditions. Although the results of Hernandez and Orioli's germination experiment differ from my findings that the small wild maternal achenes germinate most slowly, they imply that the size differences between the three maternal types of achenes studied here may be relevant to differences in their germination patterns.

In addition to determining the embryo covering structures and overall achene size, the maternal parent also plays a highly significant role in determining the size of the embryo itself. Crop-

produced embryos were the largest, followed by F1-produced embryos, followed by wild-produced embryos. Radicle width followed the same trend. Although it is tempting to view the embryo as a structure that reflects roughly equal maternal and paternal contributions because its nuclei contain roughly equal genetic contributions from both parents, this is inaccurate. Although the cross types dissected all contained approximately 50% crop alleles in the embryonic nuclei, their embryo sizes differed greatly depending on the maternal parent, and thus the size of the pericarp. This suggests that the size of the embryo is not dictated entirely by its genetic code. In fact, data from other studies show that seeds produced on the same wild maternal plants with different percent crop alleles in their embryos tended to have similar weights (Mercer and Alexander, unpublished data). Instead, the growing embryo is responsive to the maternally inherited pericarp that surrounds it. One possible explanation for this phenomenon is that the embryo grows until it hits the inner walls of the pericarp, and then it stops. Regardless of the precise developmental mechanism, embryo size appears to be a largely maternal effect. However, further study could focus on a greater variety of cross types, representing multiple levels of crop alleles within a given maternal type, to determine whether percent crop alleles is an additional factor in determining embryo size. Embryo size may have important implications for germination patterns by affecting how quickly an embryo can swell to push apart the two faces of the pericarp or how forcefully its radicle can extend.

Implications for introgression of crop alleles into wild populations

Although it does not paint a complete picture of the effects of fall germination rates on crop-wild gene flow in *H. annuus*, this project does have practical implications. If all sunflower seeds containing crop genes germinated at an inappropriate time of year and were killed by winter

weather before reproducing, crop gene introgression into wild populations would not be a serious concern. The results of this study suggest that germination and death of crop-wild hybrid seeds under fall conditions is not high enough to preclude the introgression of crop alleles into wild populations, especially if those alleles also bring positive fitness effects such as rapid growth (Mercer et al. 2007), herbicide resistance, pest resistance (Snow et al. 2003), or a wilder climatic tolerance. This study also identified several maternally inherited achene characteristics of crop-wild hybrids that could also have consequences for fitness and thus crop gene introgression. For example, seed size could be important in a hybrid zone. The larger crop- and F1 produced seeds could face increased predation risks, lower dispersal rates, or they could yield larger seedlings that have a competitive edge over their smaller neighbors. Ten out of the 14 cross types containing crop genes still had dormant seeds at the end of the 27 day germination period (Fig. 5), implying that the reduced dormancy associated with crop genes may not be significant enough to prevent the introgression of crop genes, including transgenes, into wild populations.

Seeds in crop and F_1 hulls germinated more readily in the simulated fall conditions than seeds in wild hulls, which suggests that the most probable route of crop gene introgression may be crop pollen fertilizing wild seeds. These wild-produced seeds would be most likely to overwinter in a dormant state and germinate at an appropriate time in the spring. Crop pollen may also disperse farther than crop seeds as it carried by pollinating insects. This larger dispersal range increases the chance of crop pollen contacting a wild population in a hybrid zone. This study also has implications for the movements of crop alleles once they introgress into wild populations. For example, a WxF1, is less likely to fall germinate than an F1xW, so I would expect the crop genes to preferentially disperse via hybrids pollinating wild plants. However, all pathways of crop-

gene introgression should continue to be considered. In this study, even some crop-produced achenes carrying 75% crop alleles remained dormant throughout the 27 day germination period, suggesting that seed-mediated gene flow is also possible. Although pollen generally has a higher dispersal rate than seeds, Wegier et al. (2011) have suggested that the accidental dispersal of crop seeds, including transgenic varieties, during the human-mediated transportation may be a significant route of gene flow in some crop-wild systems. If the pollen of nearby wild populations fertilizes crop populations, subsequent seed-mediated gene flow could also result in the introduction of crop-produced F_1's into wild populations.

Recurrent gene flow further complicates potential pathways of crop gene introgression. Sunflower producers often plant the same fields year after year, facilitating annual waves of crop gene introgression into nearby wild populations. In this recurrent gene flow situation, the introgression of crop alleles from the current production season likely occurs simultaneously with the dispersion of crop alleles that have already introgressed from previous production seasons. Consequently, crop fields could pollinate a variety of crop-wild hybrid cross types already living among wild populations, in addition to pollinating wild plants that contain no crop alleles.

Because this study indicates that the reduced dormancy associated with crop alleles and the maternal effects of crop- and F1-produced achenes are not a complete barrier to crop gene introgression, further research is needed on the rates of crop-wild gene flow in *H. annuus*, as well as the ecological effects, and weed management implications of hybridization. Future studies of germination and dormancy should use controlled field experiments to mimic actual hybrid zone conditions. Additional studies are also needed to determine the fitness effects of

crop alleles and maternal effects on wild plants after germination. Such fitness studies should test traditional as well as novel crop alleles under a variety of selective pressures possible in hybrid zones including competition, pests, herbicide application, soil nutrient levels, and different climatic variables. In addition to studying the effects of crop alleles in wild populations to predict the most probable routes of crop gene introgression, research should seek to quantify the rate of this introgression under realistic hybrid zone conditions. Such research is necessary to inform agricultural and land management policies.

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Tables

Table 1. Expected average percent crop alleles in seed embryo for each cross type used organized based on maternal and paternal parent identity. The seed coverings for each offspring are determined by the maternal parent.

	Maternal parent		
Paternal parent	Wild	F_1	Crop
Wild	0	25	50
Backcross	12.5	37.5	62.5
F_1	25	50	75
F_2	25	50	75
Crop	50	75	100

Table 2. Growth chamber settings designed to simulate fall weather conditions in Lawrence, KS (The Weather Channel).

	Day	Night
Length	11 hrs.	13 hrs.
Light	Yes	No
Temperature	21°C	9°C

Table 3. ANOVA to identify the effects of maternal type and percent crop alleles, as well as the maternal × percent crop alleles interaction, on days 9, 19, and 27, as calculated with SAS GLIMMIX. Significant effects ($P < 0.05$) are in bold.

	Maternal (Mat)			% Crop Alleles (CA)			Mat × CA		
Day	DF [a]	F	P	DF [a]	F	P	DF [a]	F	P
9	2,135	73.37	**<.0001**	1,135	39.84	**<.0001**	2,135	14.45	**<.0001**
19	2,135	16.19	**<.0001**	1,135	35.05	**<.0001**	2,135	16.35	**<.0001**
27	2,135	11.53	**<.0001**	1,135	13.74	**0.0003**	2,135	12.47	**<.0001**

[a] Numerator df followed by denominator df

Table 4. Arithmetic means, least squares means, and standard errors of proportion of viable seeds germinated (i.e., germinated/(total-achenes without embryos-achenes lost)) for each maternal type. Letters reflect significant differences ($P < 0.05$) for a given day, between least squares means, using Tukey-Kramer adjustment for multiple comparisons. Least squares means, standard errors, and Tukey-Kramer comparisons were produced by SAS GLIMMIX.

	Crop				F1				Wild			
Day	Arith. Mean	LS Mean	StError		Arith. Mean	LS Mean	StError		Arith. Mean	LS Mean	StError	
9	0.909	0.922	0.026	a	0.635	0.635	0.015	b	0.224	0.327	0.026	c
19	0.931	0.953	0.022	a	0.857	0.857	0.012	b	0.775	0.88	0.022	b
27	0.939	0.961	0.018	a	0.908	0.908	0.018	b	0.887	0.921	0.018	ab

Table 5. ANOVAs of proportion of viable seeds germinated, testing effects of maternal type, paternal type, and the maternal x paternal interaction, using SAS GLIMMIX. Significant effects ($P < 0.05$) are in bold.

Source	Num df	Den df	F	Significance
Day 9				
Maternal (Mat)	2	126	668.88	**<0.0001**
Paternal (Pat)	4	126	13.71	**<0.0001**
Mat × Pat	8	126	6.94	**<0.0001**
Day 19				
Mat	2	126	50.21	**<0.0001**
Pat	4	126	12.10	**<0.0001**
Mat × Pat	8	126	8.25	**<0.0001**

Table 6. Analyses for each maternal type of the relationship between percent crop alleles and proportion of viable seeds germinated. Only measurements with a significant effect of maternal × percent crop alleles (Table 5). Significance ($P < 0.05$) of percent crop alleles, from t-tests, is in bold.

Maternal	DF [a]	F	P	Intercept	% Crop Alleles
Day 9					
C	1,39	0.70	0.4079	**0.9458**	-0.00051
F1	1,39	34.27	**<.0001**	**0.3569**	**0.005849**
W	1,39	21.79	**<.0001**	**0.1317**	**0.004119**
Day 19					
C	1,39	2.80	0.1024	**0.9962**	-0.00090
F1	1,39	36.94	**<.0001**	**0.6627**	**0.004082**
W	1,39	21.83	**<.0001**	**0.6801**	**0.004211**
Day 27					
C	1,39	2.89	0.0970	**1.0022**	-0.00087
F1	1,39	29.06	**<.0001**	**0.7536**	**0.003254**
W	1,39	5.52	**0.0239**	**0.8560**	**0.001377**

[a] Numerator df followed by denominator df

Table 7. Pair-wise ANOVAs of proportion of viable seeds germinated using SAS GLIMMIX. Significant effects ($P < 0.05$) are in bold.

	Wild vs. F1, 25% vs. 50%[1]				F1 vs. Crop, 50% vs. 25%[2]			
Source	Num df	Den df	F	Significance	Num df	Den df	F	Significance
Day 9								
Maternal (Mat)	1	27	78.69	**<0.0001**	1	27	64.49	**<0.0001**
Crop alleles (CA)	1	27	2.42	0.135	1	27	9.77	**0.0042**
Mat × CA	1	27	1.05	0.3150	1	27	44.13	**<0.0001**
Day 19								
Mat	1	27	0.12	0.7297	1	27	2.62	0.1169
CA	1	27	1.02	0.3216	1	27	2.62	0.1169
Mat × CA	1	27	0.02	0.8885	1	27	31.01	**<0.0001**

[1] Cross types tested were WxF1, WxC, F1xW, and F1xF1.

[2] Cross types tested were F1xF1, F1xC, CxW, and CxF1.

Table 8. ANOVA to identify the effects of maternal type and hour, as well as the maternal × hour interaction on achene measurements, as calculated with SAS GLIMMIX. All analyses employed data from W, F1, and C maternal types, except RW, for which C and F1 were used (only one W achene germinated during the experiment. Significant effects ($P < 0.05$) are in bold.

	Maternal			Hour			Maternal × Hour		
	DF [a]	F	P	DF [a]	F	P	DF [a]	F	P
AL	2,4	430.78	**<.0001**	1,6	0.60	0.4669	2,6	4.45	0.0653
AW	2,4	181.61	**0.0001**	1,6	4.34	0.0824	2,6	0.42	0.6722
AD	2,4	183.06	**0.0001**	1,6	12.18	**0.0130**	2,6	4.02	0.0781
EL	2,4	298.03	**<.0001**	1,6	9.03	**0.0238**	2,6	6.56	**0.0309**
ED	2,4	77.44	**0.0006**	1,6	20.63	**0.0039**	2,6	5.46	**0.0445**
RW [b]	1,2	64.11	**0.0152**	--	--	--	--	--	--
DRP	2,4	4.04	0.1098	1,6	11.31	**0.0152**	2,6	6.97	**0.0272**
DRP/AL	2,4	7.26	**0.0467**	1,6	7.16	**0.0368**	2,6	6.54	**0.0311**
PGW	2,4	14.39	**0.0149**	1,6	11.11	**0.0158**	2,6	9.07	**0.0154**
PGW/AD	2,4	19.51	**0.0086**	1,6	9.69	**0.0208**	2,6	14.37	**0.0052**
PSL	2,4	29.11	**0.0041**	1,6	0.81	0.4028	2,6	5.29	**0.0474**
PSL/AL	2,4	45.95	**0.0017**	1,6	1.06	0.3419	2,6	4.93	0.0542

[a] Numerator df followed by denominator df

[b] RW was not tested for effects of hour or maternal × hour because it was only measured on germinated seeds.

Table 9. Least squares means and standard errors of achene measurements (mm) for each maternal type, calculated using SAS GLIMMIX. Letters reflect significant differences ($P < 0.05$) for a given trait, between means, using Tukey-Kramer adjustment for multiple comparisons.

	Crop			F1			Wild		
Measurement	Mean	StError		Mean	StError		Mean	StError	
AL	9.93	0.085	a	6.80	0.077	b	4.98	0.077	c
AW	6.47	0.106	a	4.14	0.102	b	2.50	0.099	c
AD	4.51	0.192	a	2.83	0.061	b	1.66	0.060	c
EL	8.83	0.256	a	5.60	0.256	b	4.02	0.076	c
ED	3.56	0.165	a	2.15	0.080	b	1.30	0.079	c
RW	2.48	0.069	a	1.63	0.081	b	0.95[a]	--	-
DRP	0.51	0.102	a	0.57	0.029	a	0.58	0.025	a
DRP/AL	0.05	0.017	a	0.08	0.005	a	0.12	0.004	b
PGW	0.26	0.044	a	0.06	0.019	b	0.00	0.018	b
PGW/AD	0.05	0.011	a	0.02	0.004	a	0.00	0.004	b
PSL	0.06	0.063	ab	0.12	0.021	a	0.29	0.019	b
PSL/AL	0.01	0.010	a	0.02	0.003	a	0.06	0.003	b

[a] Single measurement available, excluded from analysis.

Table 10. Analyses for each maternal type of the relationship between hour and individual trait values. Only measurements with a significant effect of hour or maternal × hour (Table 8). Regression equations are only presented where the effect of hour is significant. Significance ($P < 0.05$) of hour, from t-tests, is in bold.

Maternal	DF [a]	F	P	Intercept	Hour
AD					
C	1,2	1.53	0.3411	--	--
F1	1,2	21.81	**0.0429**	2.6147	0.004420
W	1,2	35.12	**0.0273**	1.5801	0.001593
EL					
C	1,2	17.84	0.0517	--	--
F1	1,2	1.50	0.3454	--	--
W	1,2	1.33	0.3676	--	--
ED					
C	1,2	5.66	0.1404	--	--
F1	1,2	8.05	0.1051	--	--
W	1,2	37.45	**0.0257**	1.2229	0.001500
DRP					
C	1,2	2.52	0.2536	--	--
F1	1,2	38.86	**0.0248**	0.7691	-0.00475
W	1,2	6.12	0.1319	--	--
DRP/AL					
C	1,2	3.22	0.2147	--	--
F1	1,2	67.58	**0.0145**	0.1160	-0.00077
W	1,2	2.60	0.2479	--	--
PGW					
C	1,2	0.34	0.6182	--	--
F1	1,2	36.54	**0.0263**	-0.01522	0.001843
W	1,2	1.68	0.3245	--	--
PGW/AD					
C	1,2	0.06	0.8288	--	--
F1	1,2	33.35	**0.0287**	-0.00510	0.000635
W	1,2	1.68	0.3245	--	--
PSL					
C	1,2	0.87	0.4489	--	--
F1	1,2	14.10	0.0642	--	--
W	1,2	5.63	0.1411	--	--

[a] Numerator df followed by denominator df

Figures

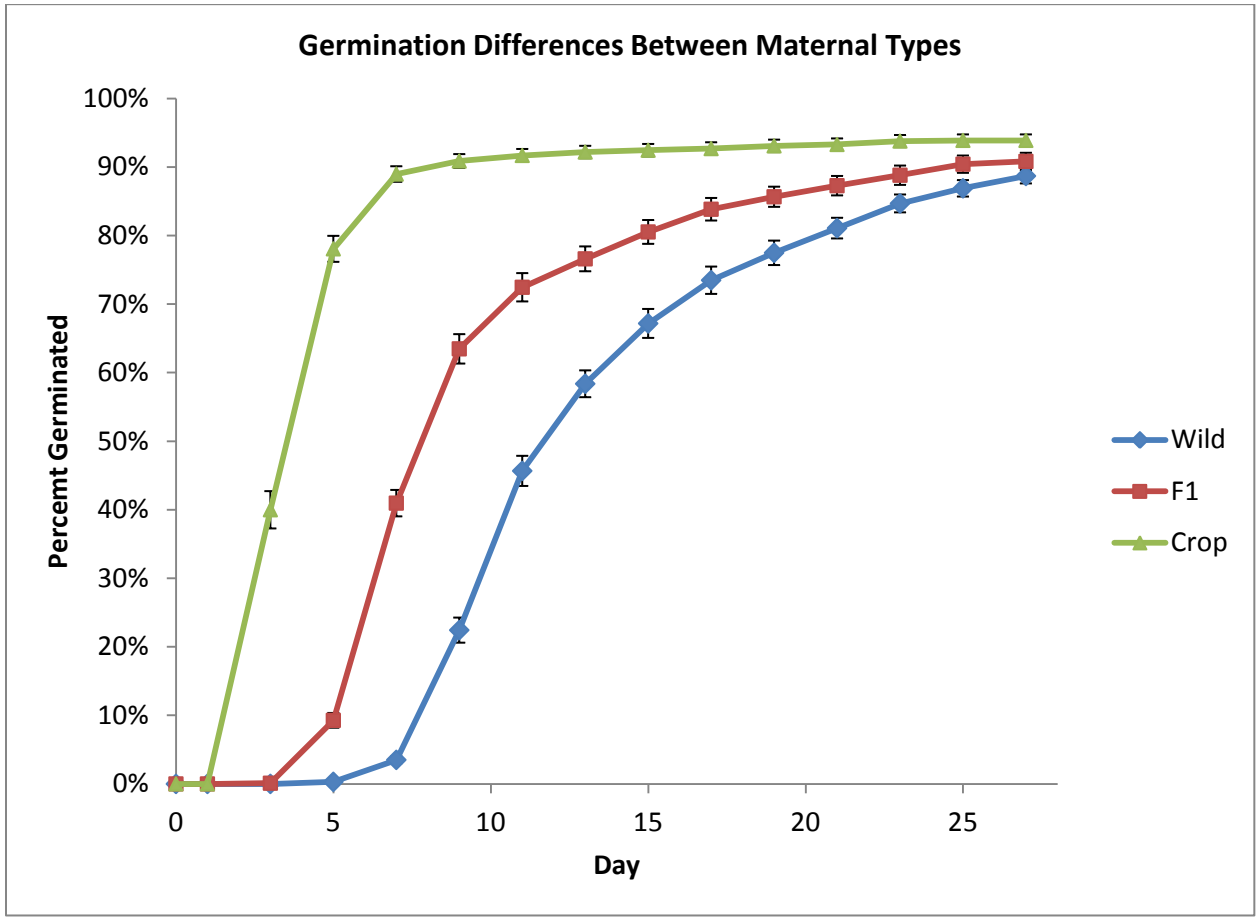


Fig. 1. A plot of the percent of viable seeds germinated (seeds germinated/(total seeds-dead seeds-lost seeds) x 100%) over time. Each series represents a different hull type. Error bars depict the standard error of the mean across the ten replications.

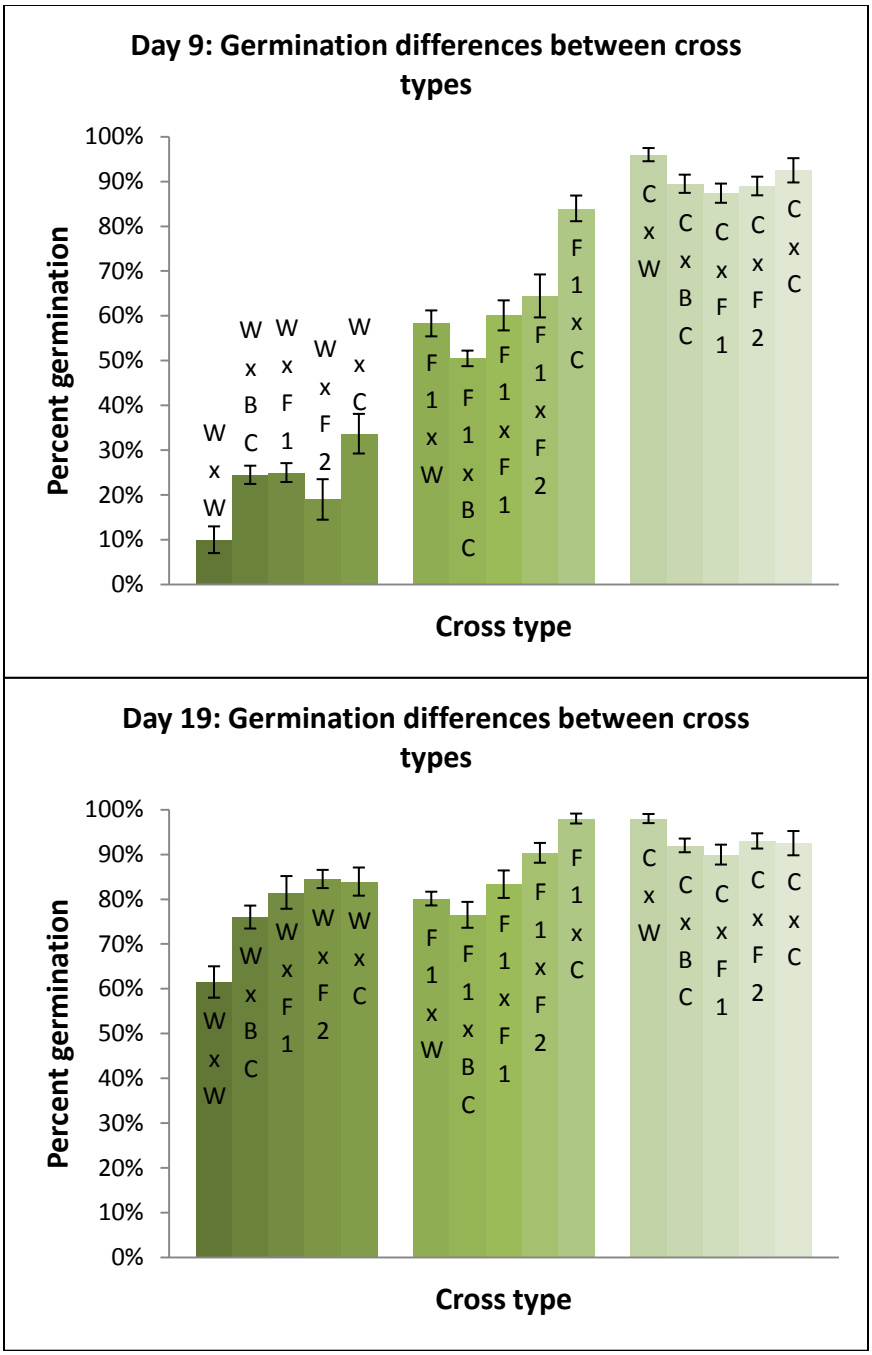


Fig. 2. Percent of viable seeds germinated (i.e. germinated/(total-achenes without embryos-achenes lost) x 100%) for each cross type after 9 (top) and 19 (bottom) days in the growth chamber. Each cluster of columns represents a maternal type, and within maternal type, the percent crop alleles in the different cross types increases from left to right. (N.b. Percent crop genes is equal for F_1 and F_2.) Error bars depict the standard error of the mean across the ten replications.

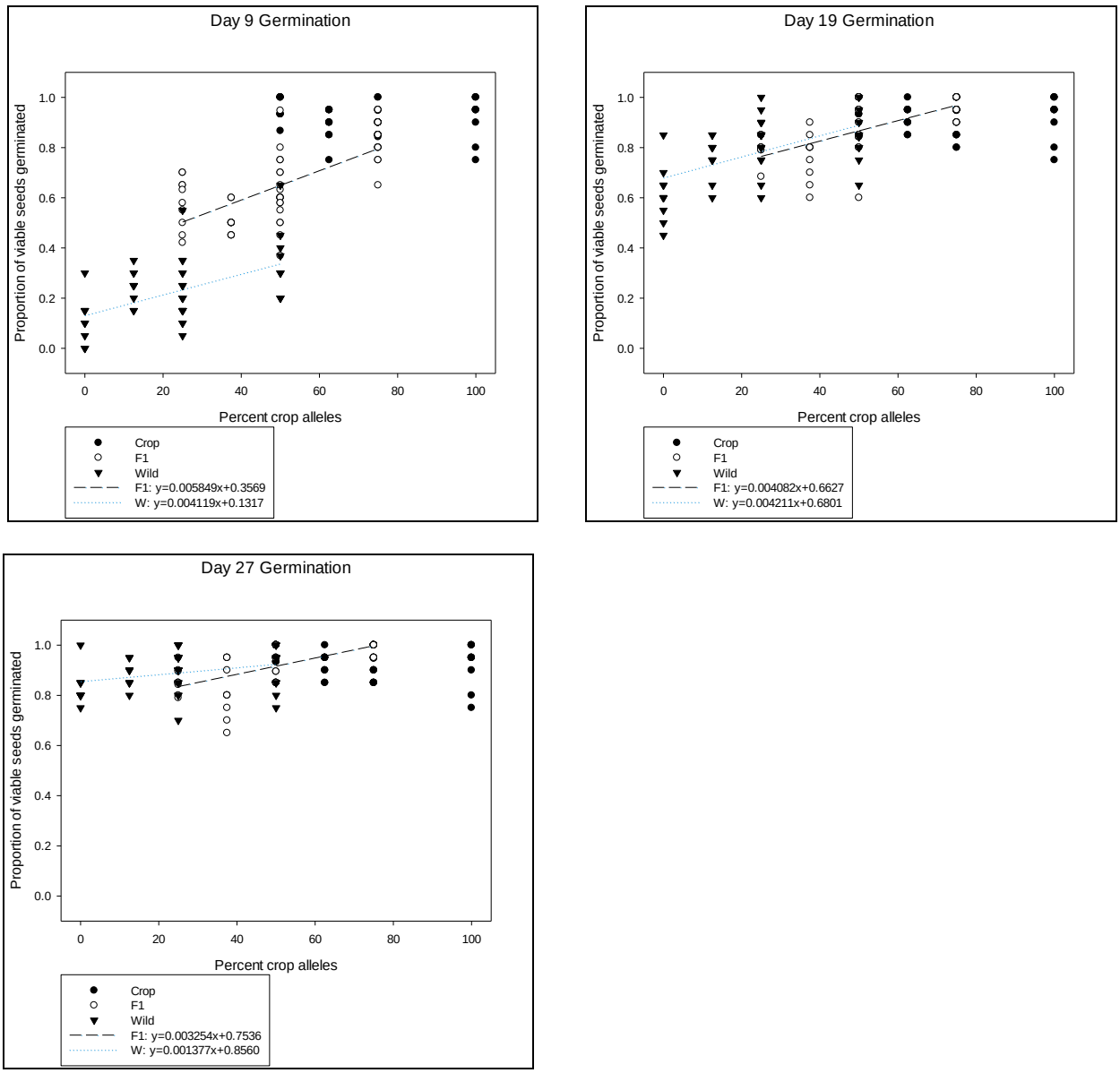


Fig. 3. Scatter plots of proportion of viable seeds germinated by percent crop alleles on days 9, 19, and 27. Significant linear regressions ($P > 0.05$ for effect of hour) by maternal type are plotted.

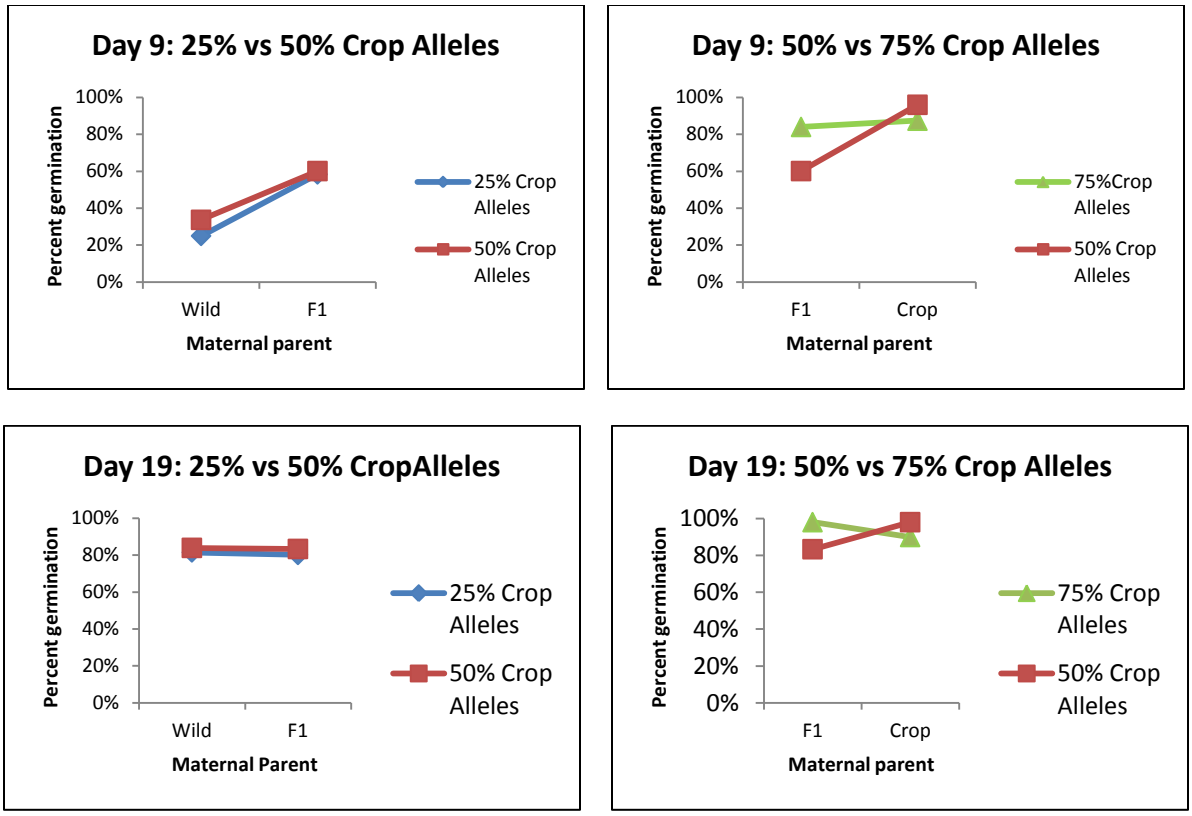


Fig. 4. Pair-wise comparisons of germination of percent viable seeds germinated on days 9 and 19 for cross types matched for maternal type or percent crop alleles.

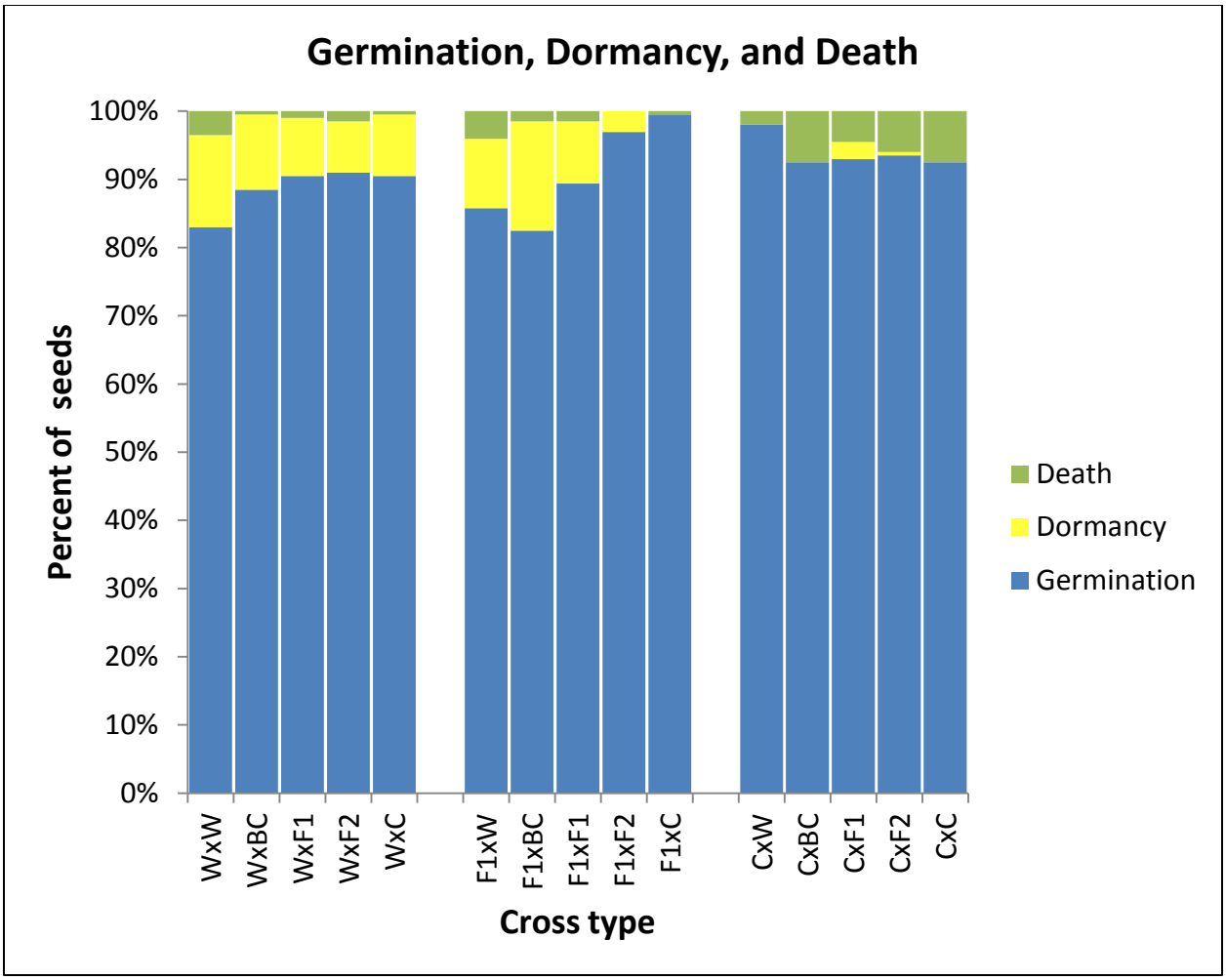


Fig. 5. The percent of total seeds studied that were germinated, dead, or dormant at the end of the study period. Each cluster of columns represents a maternal type, and within maternal type, the percent crop alleles increases from left to right. (N.B. Percent crop genes is equal for F_1 and F_2.)

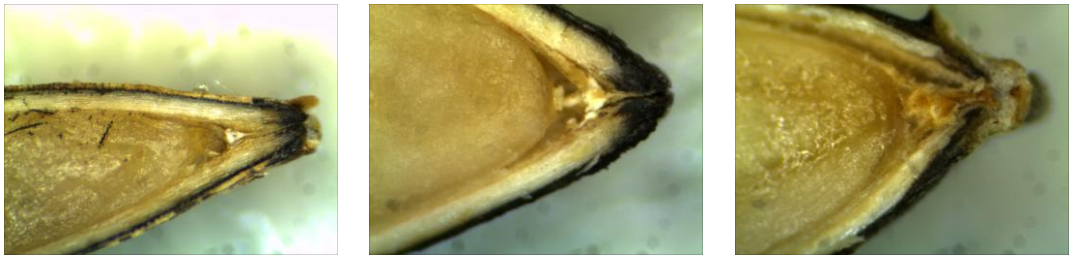
	Maternal type		
	Wild	F1	Crop
Before imbibition			
After imbibition			

Fig. 6. Cut faces of wild-, F1-, and crop-produced achenes before and after imbibition.

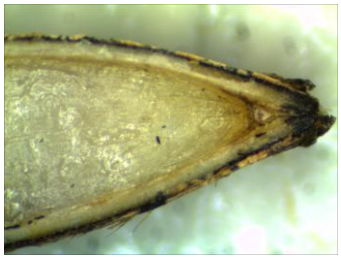
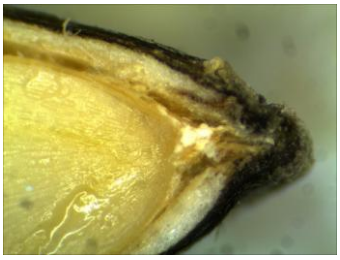
	Maternal type		
	Wild	F1	Crop
Before loose tissue dis-integration			
After loose tissue dis-integration			

Fig. 7. Cut faces of wild-, F1-, and crop-produced achenes before and after deterioration of loose tissue and the micropylar end.

	Maternal type		
	Wild	F1	Crop
Before pericarp seam splits			
After pericarp seam splits			

Fig. 8. Cut faces of wild-, F1-, and crop-produced achenes before and after pericarp seam splits. Only one crop-produced achene out of 16 on which PGW was measured had a completely closed pericarp seam (i.e., PGW = 0).

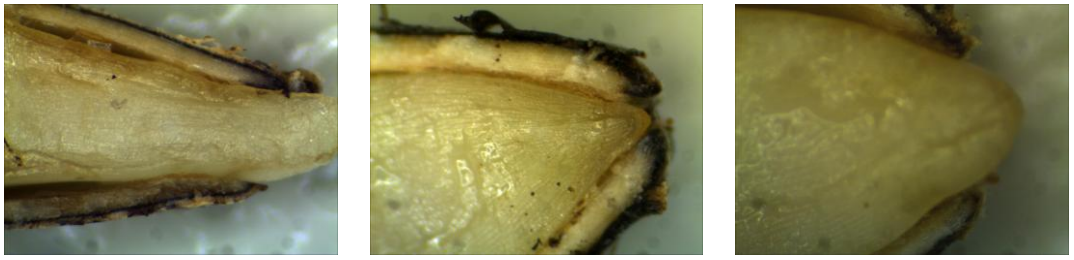
	Maternal type		
	Wild	F1	Crop
Before radicle emergence			
After radicle emergence			

Fig. 9. Cut faces of wild-, F1-, and crop-produced achenes before and after radicle emergence. Only one wild-produced achene germinated during the experiment two study period.

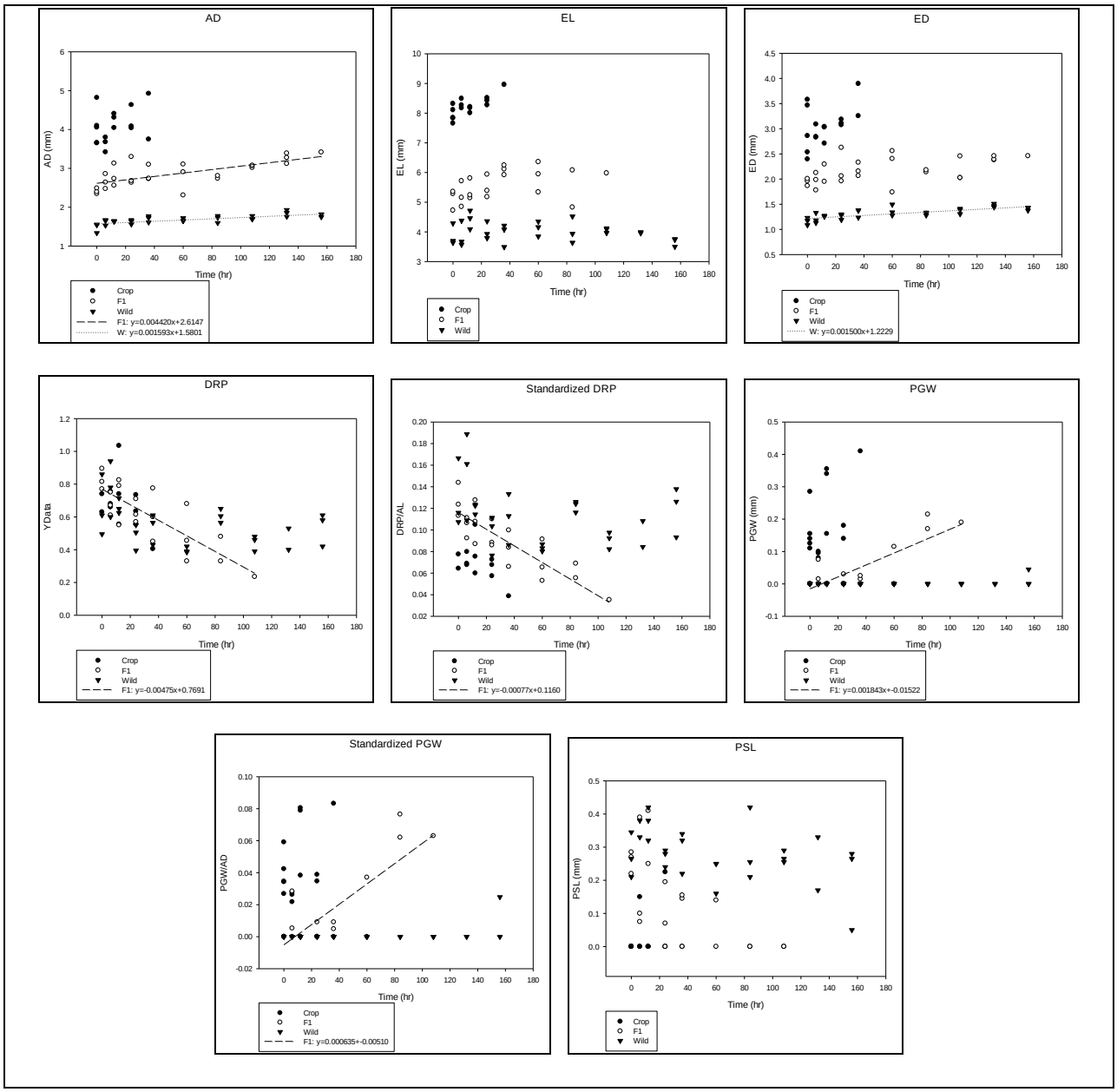


Fig. 10. Scatter plots of all measurements for which the effect of hour or the maternal × hour is significant. Trend lines are shown where the effect of hour is significant within a maternal type.

Appendix

Tables

Table S1. Number of maternal and paternal parents used in bulking, by experiment and cross type. Some crosses used bulked crop pollen, as noted. This is negligible due to genetic uniformity of the HA89 inbred line.

Cross type	Experiment 1			Experiment 2		
	One paternal parent[a]	Two paternal parents[b]	Total maternal families	One paternal parent[a]	Two paternal parents[a]	Total maternal families
WxW	0	8	8	--	--	--
WxBC	0	8	8	--	--	--
WxF1	0	8	8	--	--	--
WxF2	0	8	8	--	--	--
WxC	0	8[c]	8	0	8[c]	8
F1xW	6	2	8	--	--	--
F1xBC	1	7	8	--	--	--
F1xF1	0	8	8	0	8	8
F1xF2	5	3	8	--	--	--
F1xC	6	2[d]	8			
CxW	5	0	5	2	0	2
CxBC	3	2	5	--	--	--
CxF1	5	3	8	--	--	--
CxF2	4	4	8	--	--	--
CxC	8[e]	0	0	--	--	--

[a] Number of maternal families pollinated by only one paternal parent

[b] Number of maternal families pollinated by two paternal parents

[c] Bulked crop pollen used as both paternal parents

[d] One of the two paternal parents was bulked crop pollen

[e] Self-pollinated

Table S2. Abbreviations of measurements.

Abbreviation	Measurement
AL	Achene length
AW	Achene width
AD	Achene depth
EL	Embryo length
ED	Embryo depth
RW	Radicle width
DRP	Distance from radicle to pericarp
DRP/AL	Standardized distance from radicle to pericarp
PGW	Pericarp gap width
PGW/AD	Standardized pericarp gap width
PSL	Pericarp seam length
PSL/AL	Standardized pericarp seam length

Figures

Percent Crop Alleles

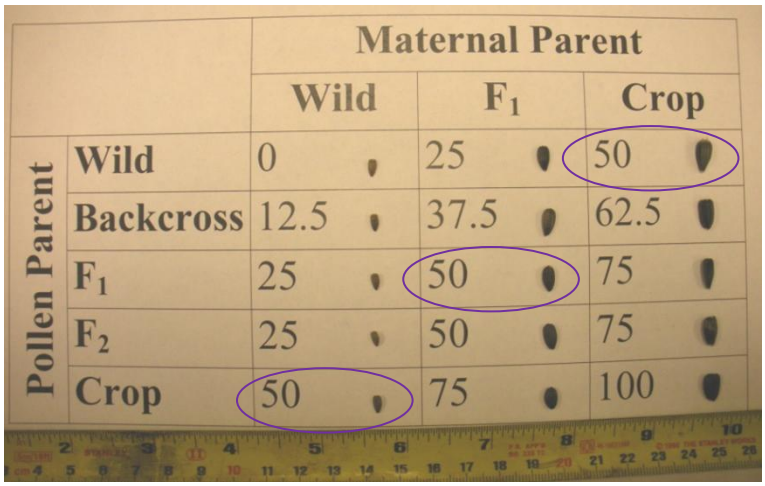


Fig. S1. A photograph of all 15 cross types used in Experiment 1, organized by parentage and percent crop genes. The three cross types used in Experiment 2 are circled.

Fig. S2. AW on a CxW achene after 0 hr.

Fig. S3. RW on a CxW achene after 60 hr.

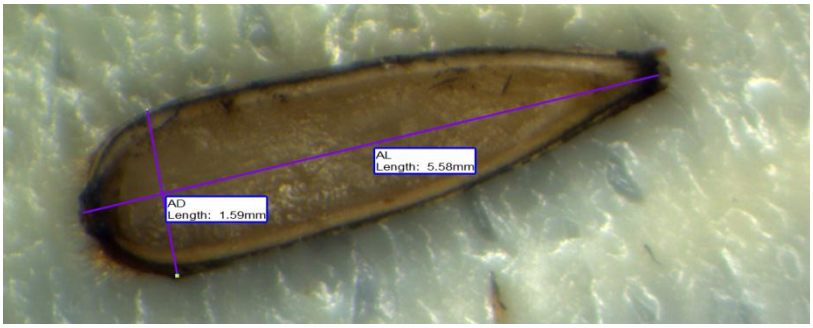


Fig. S4. AL and AD on a WxC achene after 12 hr.

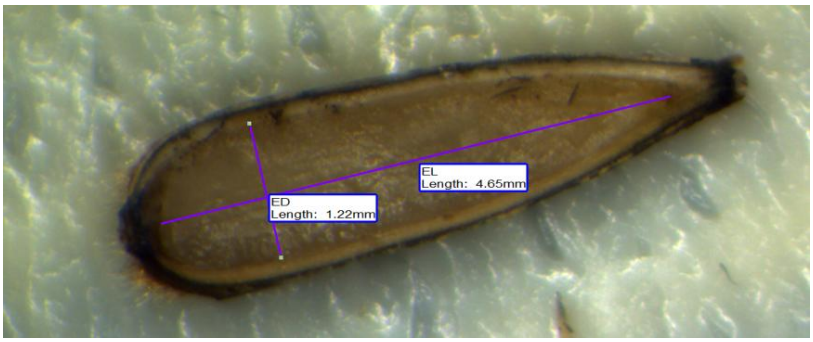


Fig. S5. EL and ED on a WxC achene after 12 hr.

Fig. S6. DRP measured on an F1xF1 after 36 hr.

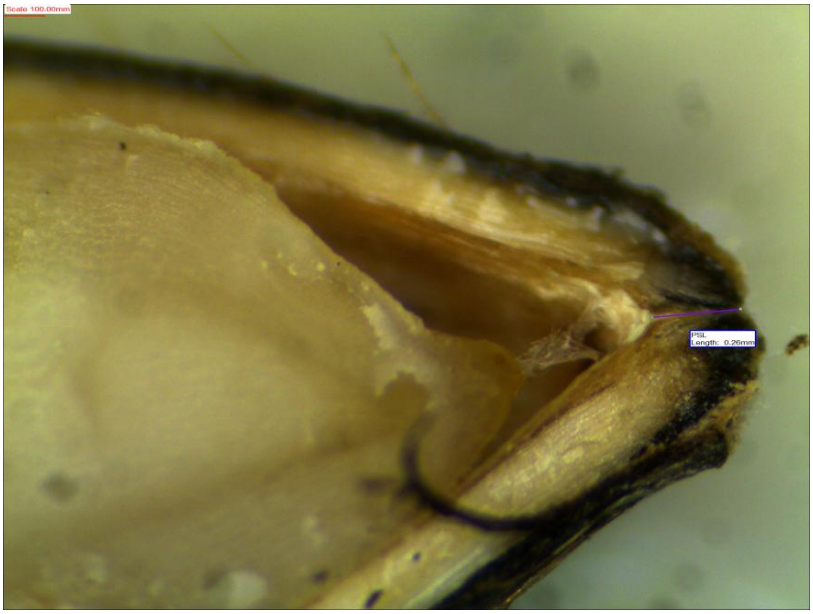


Fig. S7. PSL measured on an F1x F1 after 12 hr.

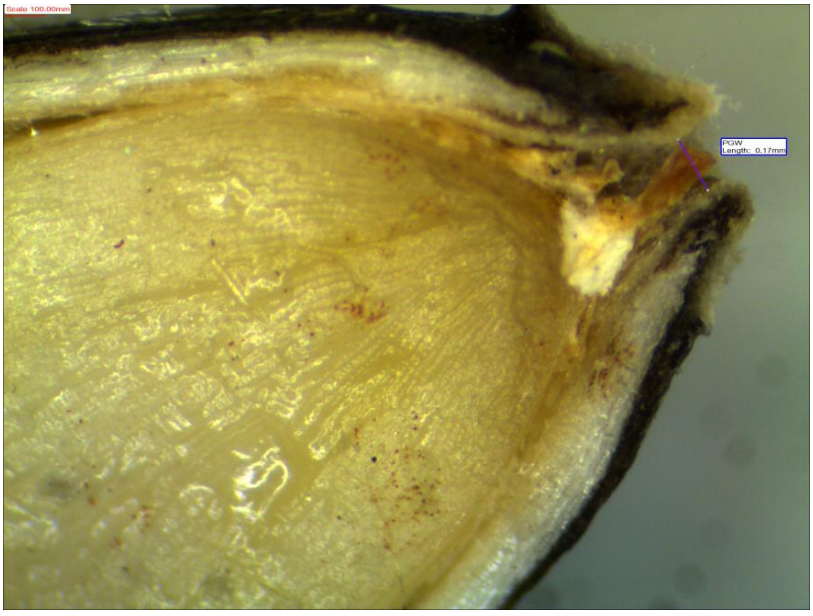


Fig. S8. PGW measured on a C x W after 24 hr.

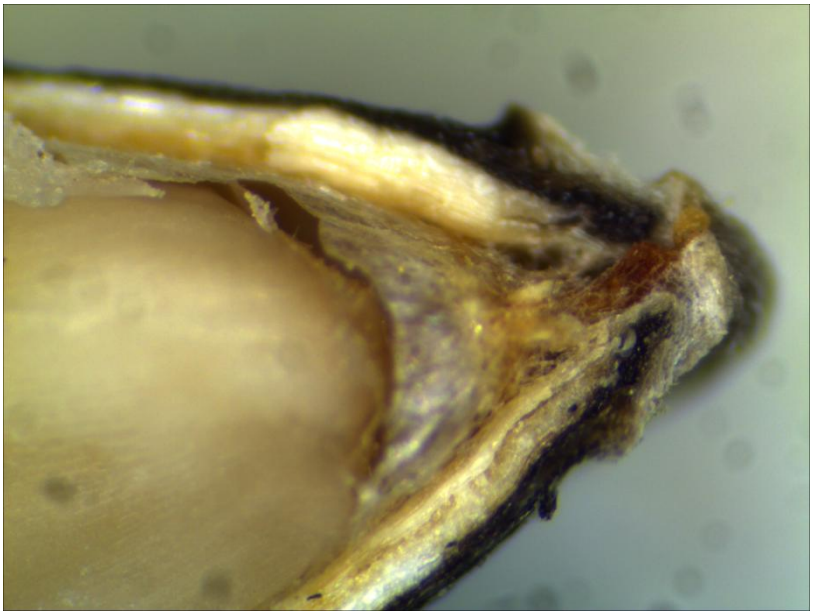


Fig. S9. The cut face of a C x W achene at hour 0. The pericarp seam is already split open, and the loose tissue appears red-brown and oxidized.

Essays

Essay S1: Possible causes of high germination in experiment one.

By the end of the 27 day germination period in experiment one, all maternal types asymptotically approached high levels of germination. While such high germination was expected for crop-produced achenes due to extensive breeding efforts to eliminate dormancy in crop lines, such high germination was not expected for F1- and wild-produced achenes (Mercer et al. 2006a). Wild achenes are known to have a higher level of dormancy which allows them to overwinter. I expected F1-produced achenes to inherit some of this dormancy.

Several experimental artifacts could be responsible for the higher than anticipated germination of F1 and wild maternal types. First of all, it may reflect differences between simulated and natural conditions. Natural temperatures fluctuate more throughout the day and vary day to day than growth chamber conditions. Soil moisture may also be more variable in a natural setting. The chemistry of soil water may differ from that of the water used in the experiment. Likewise, microbe populations in natural soils likely differ from those in experimental germination media. The combination of experimental conditions may have stratified the seeds, reducing their dormancy. Many of the environmental variables controlling sunflower seed germination are still poorly understood (Walters 2006) and may therefore be inadequately addressed when creating simulation conditions. This highlights the shortcomings of laboratory germination experiments, and the potential advantages of a controlled field experiment approach.

The unexpectedly high germination rates could also be attributable to the difference between environmental conditions in which the wild population evolved and the conditions in which the seeds were produced for this experiment. Certain aspects of the maternal environment affect seed development which may in turn influence germination patterns. For example, Lindstrom et al. (2007) demonstrated that shading maternal *H. annuus* plants resulted in achenes with thinner, lighter pericarps, and Mercer et al. (2006a) noticed differences in dormancy between sunflower seeds produced in a green house and seeds produced in a field. The controlled farm conditions and central Ohio climate in which the seeds for this experiment were produced may differ in important ways from the conditions in which the wild sunflowers have evolved in Kansas.

Perhaps the greatest factor contributing to the unexpectedly high germination rates of F1- and wild-produced achenes was the conditions under which the achenes used in this experiment were stored prior to the experiment. Between harvest and experimentation, the achenes were stored dry, in opaque paper envelopes, at room temperature. These conditions likely incited a dormancy reducing process called after-ripening. According to Finch-Savage and Leubner-Metzger (2006), after-ripening is a common process used to release dormancy, and it occurs when freshly harvested, mature seeds are stored at room temperature, usually for several months. This description exactly matches the storage conditions of the achenes used in these experiments. Effects of seed after-ripening can include germination under a wider range of temperatures and light conditions, changes in the balance of chemicals within the seed that affect germination, and increased speed of germination (Finch-Savage and Leubner-Metzger 2006).

Brunick (2007) studied the effects of after-ripening on *H. annuus* and determined that after-ripening for as little as four weeks significantly increased germination, particularly by diminishing embryo-level dormancy. The disproportionately large impact of after-ripening on embryo dormancy, as opposed to seed coat- or pericarp- imposed dormancy (Brunick, 2007) may have also diminished or otherwise altered the relationship between the percent crop alleles in the embryonic nuclei and germination, while leaving the effect of maternal parent relatively unaffected. Since the embryo is the only structure that differentiates between the different cross types within a maternal type (i.e., their seed coats and pericarps are all alike), eliminating embryo dormancy would eliminate differences in the dormancy characteristics of the different cross types within a maternal type. To avoid experimental artifacts in future studies, I suggest exposing seeds to more realistic field conditions rather than growth chamber conditions, cultivating wild and F1 plants used for seed production under conditions that more closely mimic those experienced by wild and feral plants, and beginning the germination experiment immediately after harvesting seeds or storing seeds in a vacuum between seed collection and germination.